Original Research Article

Histomorphological evaluation of cervical punch biopsies with the aid of P16 INK4A and KI 67

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Abstract

Background: Cervical cancer is the world's fourth most prevalent malignancy among women. This study examines cervical cancer incidence and mortality in India and its states over the past three decades to follow the success of preventative and control efforts. Cervical cancer is highest among 15-29-year-old women. India, HPV 16 and 18 cause it. One of the outcomes of viral genomic integration (E7) into the host cell (RB) is increased expression of p16INK4A, a cyclin-dependent kinase inhibitor. E6 suppresses p53-mediated apoptosis, prolonging the survival of injured and altered cells and raising Ki-67 (a proliferation marker). p16INK4A and Ki 67 may be employed as surrogate markers in identifying and evaluating cervical neoplasms.

Objectives
1) Study the histo-morphological features of cervical punch biopsies with the help of immune markers P16 and Ki 67.
2) To assess the utility value of p16 and ki 67 in diagnosing and grading the cervical neoplasm.

Materials and Methods: The study included 80 cervical punch biopsy specimens (22 normal cervical tissue samples, 25 low-grade squamous intraepithelial lesions (LSIL), 13 high-grade squamous intraepithelial neoplasia lesions (HSIL), 17 squamous cell carcinomas and 3 adenocarcinomas). 80 formalin-fixed, paraffin-embedded, H&E-stained tissue slides were histopathologically evaluated. After antigen retrieval, p16INK4A expression and Ki 67 were analysed using mouse monoclonal antibodies. P16INK4A staining was scored 0-8 and Ki 67 0-3. Statistical analysis was done.

Results: P16INK4A immunoreactivity was absent in all normal cervical tissues examined and Ki 67 was basally positive. There is upregulation of these biomarkers in SILs and cervical carcinoma. 16/25 cases of CIN I (LSIL) showed positivity of p16, 19/25 for ki 67. All cases of HSIL (10/10 CIN II and 3/3 CIN III) and invasive cervical carcinoma (17/17SCC and 3/3 adenocarcinoma) were positive for p16INK4A expression and Ki 67 and exhibited a higher score.

Conclusion: These studies indicate that p16INK4a and Ki 67 are specific biomarkers that can detect dysplastic and malignant cervical epithelium in sections of cervical biopsy samples, which helps in the identifying and grading of cervical neoplasms.

Keywords: Cervical cancer, pathology, prognostic factor, carcinoma, P16 INK4A, KI 67

Introduction

Cancer of the cervix is one of the most common forms of the disease that affects the female reproductive tract around 5% of all cancer-related fatalities in females around the world. In India, females between the ages of 15 and 44 years old have a higher risk of developing cervical cancer than any other type of cancer. It is the second most commonly diagnosed form of carcinoma in females around the world, and in 2018, it was responsible for 569,847 new cases as well as 311,400 fatalities [1]. It is generally agreed that an ongoing infection with a high-risk human papillomavirus (hrHPV) is the key factor in the development of cervical cancer. hrHPV have been associated to wherever from 90 to 99.7 percent of instances of cervical cancer [2, 3]. There are only two types of the human papillomavirus that are related for 71 percent of all occurrences of cervical cancer [4]. Only in 10-15 percent of these cases does a persistent infection develop, which can eventually lead to the development of cancer (in 1 percent of cases of infection with hr-HPV), if it is not timely and appropriately managed and treated. In most cases, the HPV infections are cleared by the host immune system within one to two years. However, in some cases, the infection can linger for longer than that. It is widely accepted that HPV is one of the primary agents responsible for the development of cervical neoplasia and invasive cervical cancer [2]. The expression of important proteins like Ki-67 and p16, which are both involved in the regulation of the cell cycle, is messed up by oncogenic HPV viruses [4].

Assessment methods include the Papanicolaou (Pap) smear, ocular inspection, and human papillomavirus deoxyribonucleic acid (DNA) testing, amongst others. If necessary, diagnostic confirmation procedures such as colposcopy and biopsy are then performed. In many countries, cervical intraepithelial neoplasia grade 1 is treated conservatively and followed up with screening, whereas high-grade cervical intraepithelial neoplasia grades 2 and 3 require further intervention and treatment. Treatment plans are often established based on tissue from a cervical biopsy that has been stained with hematoxylin and eosin. Nevertheless, despite the fact that there are stringent criteria for the diagnosis of cervical intraepithelial neoplasia [5-7], there is a large amount of discordance among pathologists regarding the diagnostic interpretation of hematoxylin and eosin-stained cervical tissue. As a result of this, the influence of an incorrect pathology diagnosis on the management of the patient may be significant. During an infection caused by HPV, the viral gene E7 will connect to the RB protein, which will then disable its activity. Because of this, there is an increase in the amount of p16 protein, which is a tumour suppressor gene. Normally, RB works to limit the transcription of p16.3. Therefore, increased expression of P16 will make it possible to precisely identify dysplastic lesions. A prolonged lifespan of cells that are extensively damaged and altered, leading to an increase in protein Ki-67, is caused by the sequence E6, which suppresses apoptosis that is mediated by p53 (a proliferation marker). It has been established that immunohistochemical screening for p16INK4a can improve the inter-observer reliability of histologic diagnosis of cervical intraepithelial neoplasia [8]. Overexpression of p16INK4a in the cervix can serve as a marker for the carcinogenic activity of high-risk human papillomavirus infection [8]. Because of this, p16INK4a immunohistochemical staining can considerably increase the accuracy of cervical pre-cancer histologic diagnosis and cut down on both false-negative and false-positive biopsy results [9, 10]. The investigation of prospective biomarkers will not only allow for the comparison of the efficiency of different human papillomavirus (HPV) vaccine techniques, but it will also assist in the discovery of new pathways that are implicated in the pathogenesis of cervical dyskaryosis [5]. The identification of cervical cancer may see significant improvements in accuracy, precision, and sensitivity if specific biomarkers of dysplasia were used in conjunction with cytological or histological methods. To prevent overtreatment of false-positive cases and undertreatment of false-negative cases, the primary interpretative

categories include differentiating normal from dysplasia (CIN) of any grade and low-grade (CIN1) lesions from high-grade (CIN 2/3) lesions. These distinctions are made to classify lesions according to their grades. In addition, this is clinically relevant because, on the one hand, many low-grade lesions have the potential to spontaneously regress (reviewed by Schiffman *et al.* [5] and on the other hand, ablation therapy has been shown to have a potentially negative impact on the reproductive outcome of women [8]. Both of these factors are discussed in detail in the following paragraphs. Therefore, the purpose of this study is to evaluate the function that p-16 and ki-67 indicators play in identifying normal, CINs, and carcinoma, as well as to analyse whether or not these markers could be employed as diagnostic adjuncts.

Objectives

1. To study the histo-morphological features of cervical punch biopsies with the help of immune markers P16 and Ki 67.
2. To assess the utility value of P16 and Ki 67 in diagnosing and grading the cervical neoplasm.

Materials and Methods

In the current study, particular emphasis is placed on the role that immunohistochemistry (namely p16 and ki 67) plays in premalignant and malignant cervical lesions. This one is a retrospective, study that was carried out on random samples of 80 cervical punch biopsy specimens (cases and controls) that were sent to the Department of Pathology from Mallareddy Medical College for Women. These hospitals are located in Hyderabad, India. After obtaining ethical clearance for the utilization of all samples, this investigation was carried out between January 2018 and November 2021.

Inclusion requirements

Histopathological tissues were collected from female patients between the years 2018 and 2021 who presented with cervicovaginal discharge and a cervix that was unhealthy. Patients ranged in age from 20 to 70 years old.

Exclusion criteria

1) A recorded case of cervical cancer (either untreated, in the process of treatment, or after treatment) *Initially, in the synopsis, we omitted the fact that we had eliminated squamous cell carcinomas inadvertently; however, later on, we included them in our study as positive controls.
2) Tissue sections that lacked appropriate study material and displayed significant necrosis and haemorrhage were omitted from the research.

After being properly fixed in formalin at a concentration of 10% upon their arrival at the department, the specimens were next grossly examined following the standard operating procedure for cervical biopsy specimens. The complete tissue sample obtained from the cervical biopsy was then embedded in paraffin after going through the standard processing steps. Sections that had been stained with H&E were evaluated, and slides that showed features of dysplasia and/or neoplasia were classified according to the FIGO Classification as cervical intraepithelial neoplasia (CIN)-CIN I/Low-Grade Squamous Intraepithelial Lesion (LSIL), CIN II, III/High-Grade Squamous Intraepithelial Lesion (HSIL), (Squamous cell

carcinomas, adenocarcinomas). Using the BioGenex Life systems histology kit following the instructions provided by the manufacturer, immunohistochemical staining for p16INK4A and KI 67 was performed on a total of 22 cases of chronic cervicitis, 38 cases of CIN lesions, 17 cases of SCC and 3 adenocarcinomas. These cases were studied collectively. Because it is assumed that a normal cervix will not express p16INK4A, p16INK4A was looked for in each of the 27 random normal cervical samples to verify the absence of a link between the two. In addition, 17 cases of SCC that were very clearly visible in the H and E sections were used as a positive control to verify the positive link between the two. In the immunohistochemistry investigation, the sections of the tissue that had severe necrosis or haemorrhage and insufficient viable tissue were omitted from consideration.

Immunohistochemical method

The horseradish peroxidase method was used to perform the immunohistochemistry (IHC) on sections of tissue that were embedded in paraffin and had been fixed with 10% formalin. The sections had a thickness of 3 micrometers and the IHC was performed using a panel of 2 antibodies (p16 and Ki-67; further information can be found in the table below).

Interpretation of p16ink4a staining-semiquantitative

Nuclear and/or both nuclear and cytoplasmic staining was read as positive for p16; cytoplasmic staining alone was considered nonspecific and recorded as negative. Positive results for p16 required either nuclear or both nuclear and cytoplasmic staining [7].

Interpretation of Ki 67 staining

The sections that were stained for the Ki-67 proliferation index (also known as nuclear staining) were given a score between 1 and 3 based on the following criteria: '+' indicates mild proliferation (10–30 percent positive cells); '++' indicates moderate proliferation (30–50 percent positive cells), and '+++' indicates strong proliferation (50-100 percent positive cells) (more than 50 percent positive cells). 51 minus 10 percent positive cells; the baseline positivity is considered to be 0. Negative.

Table 1: Presents the scoring method for the KI-67 staining [8]

Staining	Score
Less than 10% positive staining/Basal positivity	0
10-30% positive staining	1
30-50% positive staining	2
More than 50% positive staining	3

Analysis based on statistics

To describe the findings of the investigation, descriptive statistics were utilised. In the past, histological diagnosis was regarded as the gold standard. To determine the sensitivity and specificity of the immunohistochemical tests for p16 INK4a and Ki-67, which were used for the detection of squamous intraepithelial lesions, the performance of these tests was examined with the help of standard contingency tables. It was determined that the P value was significant if it was less than 0.01 using either the Fisher exact test or the CHI square test.

Results

Next to endometrial biopsies, cervical biopsies continue to make up the vast majority of gynecologic specimens that are obtained through the gynaecology department's biopsy procedures. Eighty patients were taken into consideration for this investigation. The range of ages covered by this group was from 21 years (CIN II) to 70 years (SCC). The age range between 41 and 60 years old has the highest prevalence of cancer in women aged 31 to 40 years old, chronic cervicitis and LSIL infection rates are at their highest. HSIL, SCC, and adenocarcinoma were prevalent in individuals aged 41 to 60.

Table 2: Age distribution of patients

Age in years	Normal	LSIL/CINI	HSIL (CINII, III)	SCC	Adeno CA	Total
21-30	5	4	1	0	0	10 (12.5%)
31-40	10	13	3	5	0	31 (38.75%)
41-50	2	4	2	4	0	12(15%)
51-60	3	3	5	6	1	18(22.5%)
61-70	2	1	2	2	2	09 (11.25%)
Total	22	25	13	17	3	80 (100%)

CIN-Cervical intraepithelial neoplasm, LSIL-Low grade squamous intraepithelial lesion, HSIL-High grade intraepithelial lesion, SCC-Squamous cell carcinoma, Adeno CA-Adenocarcinoma.

In total, eighty different cervical tissues were used for the research. Of the 80 cases, 22 were histologically diagnosed as normal or chronic cervicitis (10 with squamous metaplasia and 2 with immature squamous metaplasia) and 38 cases were diagnosed as Squamous Intraepithelial lesions (25 CIN I /LSIL, 10CIN II/HSIL (3 with squamous metaplasia) and 3-CIN III/HSIL). The remaining 20patients were classified as invasive carcinomas and included 17 cases of squamous cell carcinoma (7 keratinizing and 10 non-keratinizing) and 3 cases of adenocarcinoma.

Table 3: Distribution of cases and controls

Histological types	Number of cases	Percentage of cases
Chronic cervicitis	22 (with SM-12, with ISM-2)	27.5%
LSIL (CIN I)	25 (3 with SM)	31.25%
HSIL (CIN II)	10	12.5%
HSIL (CIN III)	3	3.75%
SCC	17	21.25%
ADENOCa	3	3.75%

Immunohistochemical analysis of p16 staining

Biopsies containing normal tissues, CIN1, CIN2, CIN3 and invasive cervical carcinoma (ICC), evaluated using the immunohistological score (0-8).

Table 4: Immunohistochemical analysis of p16^{INK4A} expression in 80 cervix

Cervical lesions	No. of cases	IHC-P16 INK4A Score							
		0 (%)	2 (%)	3 (%)	4 (%)	5 (%)	6 (%)	7 (%)	8 (%)
Normal	22	20 (90.90%)	2 (9.09%)	0	0	0	0	0	0
CIN I	25	0	1 (4%)	4 (16%)	4 (16%)	7 (28%)	9 (36%)	0	0

CIN II	10	0	0	0	0	2 (20%)	4 (40%)	3 (30%)	1 (10%)
CIN III	3	0	0	0	0	0	0	0	3 100%
SCC	17	0	0	0	0	0	0	2 (13.33%)	13 (86.66%)
ADENOCA	3	0	0	0	0	0	0	0	3 (100%)

IHC-Immunohistochemistry, CIN-Cervical intraepithelial lesion, SCC-Squamous cell carcinoma, ADENOCA-Adenocarcinoma.

In our investigation, we did not find even a single sample that had exceptionally nuclear staining, which brings up an interesting point about the location of the staining. Few cases (2/22) of chronic cervicitis and one case of LSIL/CIN I showed only weak cytoplasmic staining and are considered nonspecific and immunoregulative. Both nuclear and cytoplasmic activity was observed in 25 cases of LSIL and all cases of HSIL and carcinomas. Among the 22 cases of chronic cervicitis, two showed focal basal weak staining, while the remaining 20 did not take up the stain, yielding a score of 0-2. Negative results were observed in squamous metaplastic and immature squamous metaplastic cells. 9 cases of CIN I were assigned a 2-4 score (one case had focal weak nuclear and cytoplasmic staining and was assigned a score of 2), and 16 cases were assigned a 5-6 score. Seven of the ten CIN II cases received a score of 6-7, two received a score of 5, and one received an 8. All CIN III (3 cases), Adenocarcinoma (3 cases), and the majority of Squamous cell carcinoma (13/20 cases) have a score of 8. Two SCC cases received a score of 7.

The semi-quantitative p16INK4A expression scoring system was simple to use and provided a more detailed picture of the variable positive staining seen in neoplastic lesions. This immunohistological score, however, did not allow for the identification of an absolute cut-off point for p16INK4A expression concerning dysplasia or cancer. As a result, we classified the scores as positive, equivocal, or negative.

Score (0-8): 3-Negative, 4-equivocal, 5-8-Positive.

Table 5: Categorisation of P16 INK4A scores as positive, equivocal and negative

Cervical lesions	Total cases	IHC –P16 INK Staining		
		Negative (<=3)	Equivocal (4)	Positive (5-8)
Normal (negative control)	22	22	0	0
	100%	100%	0%	0%
CIN I (LSIL)	25	6	3	16
	100%	24%	12%	64%
CIN II (HSIL)	10	0	0	10
	100%	0%	0%	100%
CIN III (HSIL)	3	0	0	3
	100%	0%	0%	100%
SCC (positive control)	17	0	0	17
	100%	0%	0%	100%
AdenoCa	3	0	0	3
	100%	0%	0%	100%

IHC-Immunohistochemistry, CIN-Cervical intraepithelial Lesion, SCC-Squamous cell carcinoma, ADENOCA-Adenocarcinoma.

The immunostaining was negative in all 22 cases of chronic cervicitis (100%) used as a negative control. p16 positivity was found in 64 percent (16 cases) of CIN I cases. 12 percent

(3 cases) were ambiguous, unable to categorize as positive or negative. Twenty four percent (6 cases) tested negative for p16. All CIN II cases (100%) tested positive for p16. P16 immunopositivity was found in all CIN III cases. P16 immunostaining was found in all cases of squamous cell carcinoma, both nuclear and cytoplasmic. P16 expression was found in 100 percent of adenocarcinoma cases.

Containing normal tissues, CIN1, CIN2, CIN3 and invasive cervical carcinoma (ICC), evaluated using the immunohistological score (0-3).

Table 6: Immunohistochemical analysis of KI 67 in 80 cervix biopsies

Cervical Lesions	Total Cases	IHC Staining –KI 67			
		0 (<10%)	1(10-30%)	2 (30-50%)	3 (>50%)
		Negative	Positive	Positive	Positive
Normal	22	20	2	0	0
	100%	90%	10%	0%	0%
CIN 1 (LSIL)	25	6	16	3	0
	100%	24 %	64%	12%	0%
CIN II (HSIL)	10	0	0	4	6
	100%	0%	0%	40 %	60%
CINIII (HSIL)	3	0	0	0	3
	100%	0%	0%	0%	100%
SCC	17	0	0	0	17
	100%	0%	0%	0%	100%
ADENOCA	3	0	0	1	2
	100%	0%	0%	33%	67%

IHC-Immunohistochemistry, CIN-Cervical intraepithelial lesion, SCC-Squamous cell carcinoma, ADENOCA-Adenocarcinoma.

Among 22 cases of cervicitis, 90% were negative (score 0) and showed normal basal positivity (10% positive cells), while 10% of cervicitis with hyperplastic ectocervix showed 10-30% positive cells (score 1). Squamous metaplastic and immature squamous metaplastic cells yielded negative results. Ki 67 was found to be negative in 24% of CIN I patients. In 64 percent of cases, 10-30% positivity was observed (score 1). 12 percent is less than a score of 2. (30-50 percent positivity). 40 percent of CIN II cases tested positive between 30 and 50 percent of the time, while 60 percent tested positive more than 50 percent of the time.

All SCC cases tested positive more than half of the time. In 33% of adenocarcinoma cases, 30-50% of cells were positive, and in 67% of cases, more than 50% of cells were positive.

Table 7: Correlation of positive cases of IHC (p16 and ki 67) with the histologic types

Cervical lesions Histopathological type	Immunohistochemistry-Positivity	
	P 16 INK 4A	KI 67
Chronic cervicitis (n=19)	0/22	2/22
CIN I (n=25)	16/25	18/25
CIN II (n=10)	10/10	10/10
CIN III (n=3)	3/3	3/3
SCC (n=17)	17/17	17/17
Adenocarcinoma (n=3)	3/3	3/3

All 22 cases of cervicitis tested negative for p16 and ki 67. (except for 2 cases with hyperplastic ectocervix which showed 10-30 percent ki 67 positivity). Out of 25 LSIL cases, 16 had p16 positivity and 18 had ki 67 positivity. Thus, three p16 negative CIN I cases had increased Ki 67 expression. HSIL (CIN II (10/10 and CIN III (3/3), Squamous cell carcinoma (17/17) and adenocarcinoma (3/3) all showed p16 and ki 67 positivity and correlated well with histopathology.

Table 8: Sensitivity and Specificity of immune markers-p16 and ki 67

Tests	Specificity	Sensitivity				
		CIN1	CIN II	CIN III	SCC	AdenoCa
P16	100%	65%	100%	100%	100%	100%
Ki 67	90%	75%	100%	100%	100%	100%

The specificity of p16 is 100% and that of ki 65 is 90%. The sensitivity of p16 and ki 67 in picking up LSIL is 65 % and 75% respectively, whereas, in the case of HSIL and carcinomas, both showed 100% sensitivity.

Table 9: Association of immunoscore between normal and LSIL, HSIL, SCC and Adenocarcinoma

Markers	Normal			
	LSIL	HSIL	SCC	AdenoCa
P16	P(<0.01)	P(<0.01)	P(<0.01)	P(<0.01)
Ki 67	P(<0.01)	P(<0.01)	P(<0.01)	P(<0.01)

In our study, a statistically significant association was observed between normal cervix and SILs and carcinomas for both p16^{INK4a} expression and ki 67 ($p<0.01$). However, these markers are not statistically significant between LSIL and HSIL p(0.023).

Photographs taken during our study period CINI/LSIL
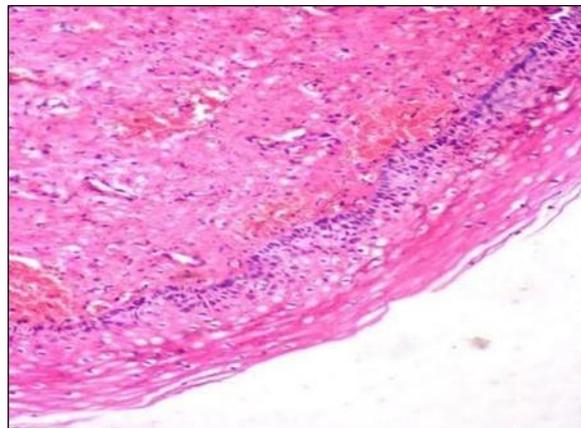
Fig 1: H and E: Koilocytes and atypia in the lower 1/3 rd of the epithelium (10 X)
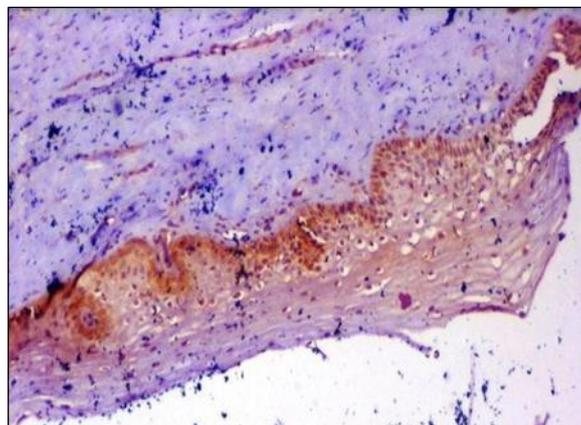
Fig 2: P 16: Moderate staining (lower 2/3rd), nuclear and cytoplasm (10 X)
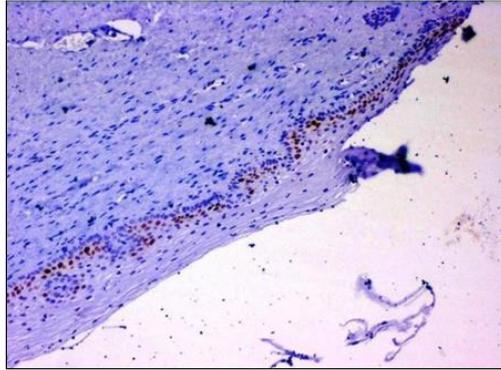
Fig 3: Ki 67: Positivity in basal and suprabasal layers, score 1 (30%)

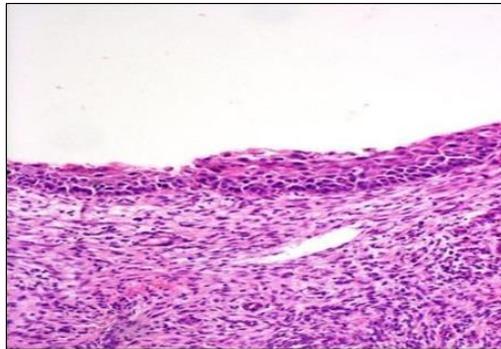**CIN II/HSIL**

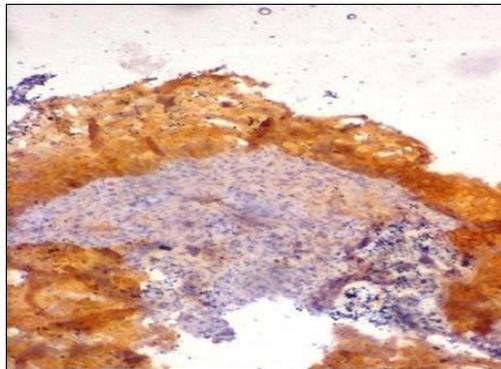**Fig 4:** H and E: Atypia > 1/2 of the epithelium (20 X)

Fig 5: P 16: Strong, diffuse positivity (score 8), nuclear and cytoplasm (10 X)
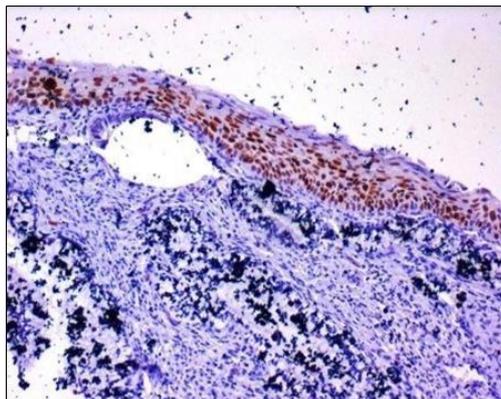

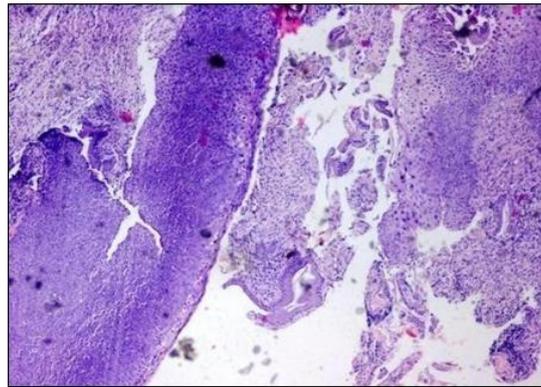**Fig 6:** Ki 67: 70% positivity, score 3 (10 X)

CIN III/HSIL

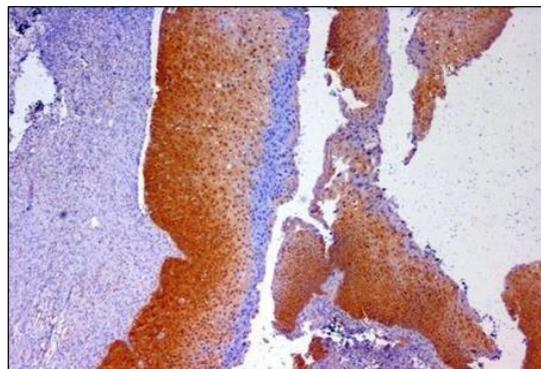**Fig 7:** H and E: Atypia > lower 2/3rd of the epithelium (10 X)

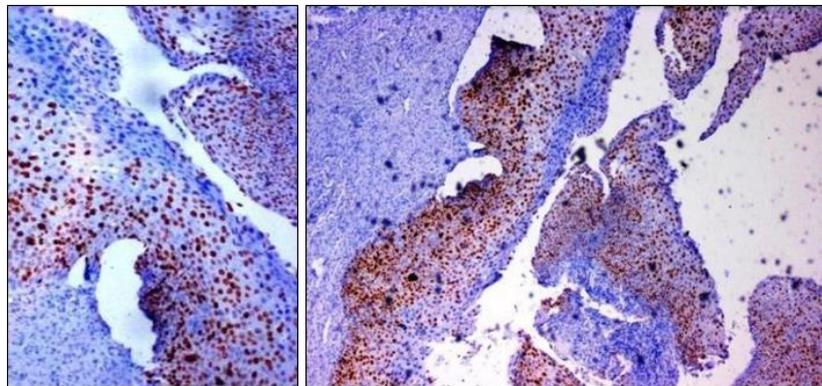**Fig 8:** Strong, diffuse positivity, nuclear and cytoplasm, score 8 (10 X)

Fig 9: Ki 67: 80% positvity, score 3 (10 X) (20 X)

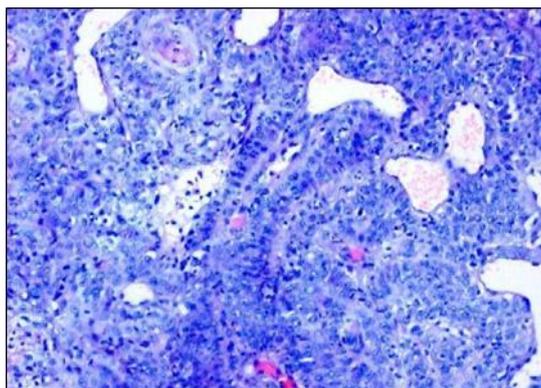**Squamous cell carcinoma (positive control)**

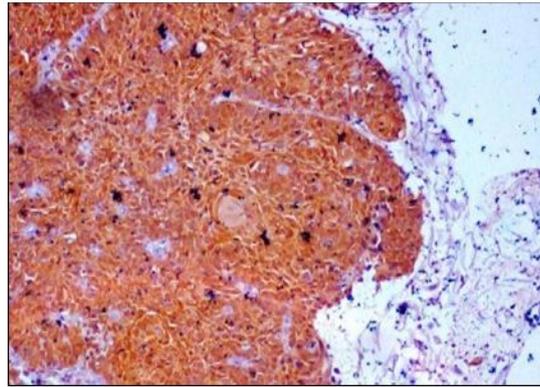**Fig 10:** H and E: Diffuse sheets of neoplastic squamous cells (40 X)

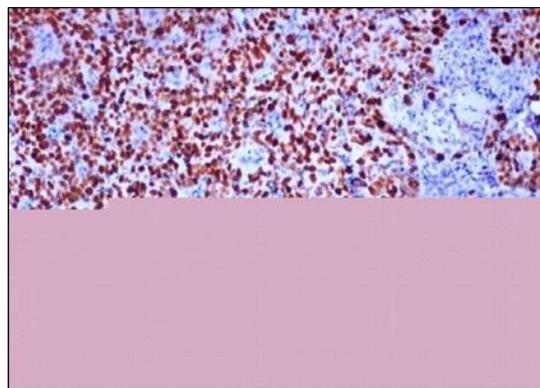**Fig 11:** P 16: Strong, diffuse positivity, nuclear and cytoplasm, score 8 (40 X)

Fig 12: Ki 67: 95% positivity, score 3 (40 X)

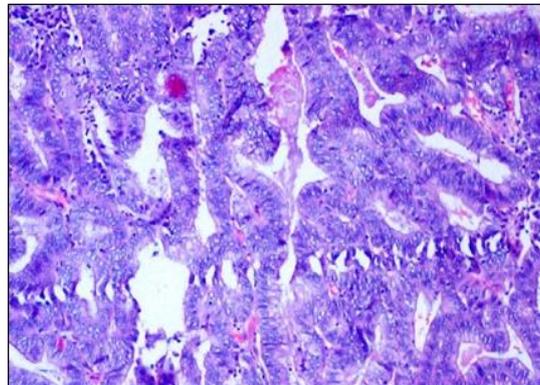**Adenocarcinoma**

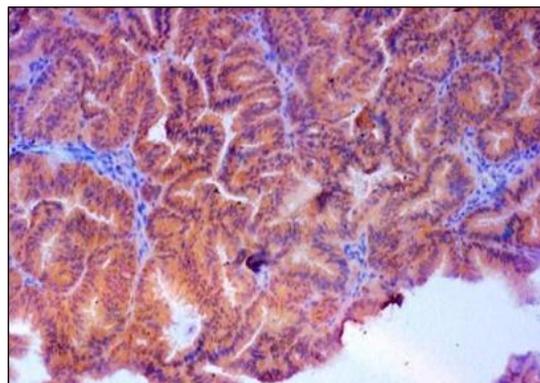**Fig 13:** H and E: Glands with neoplastic cells (40 X)

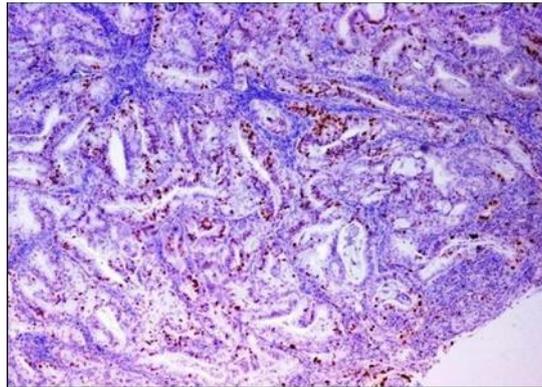**Fig 14:** P 16: Strong, diffuse positivity, score 8 (20 X)

Fig 15: Ki 67: 80% positivity, score 3 (20 X)

Discussion

The human papillomavirus (HPV) is a series of tiny DNA viruses that can be subdivided into two categories: the "low risk" (types 6 and 11) and the "high risk." Types 6 and 11 are the "low risk" variants (type 16 and 18). There is evidence that viruses associated with high risk are involved in the pathogenesis of cervical neoplasia. The progression to cervical cancer caused by HPV involves several stages. However, other risk factors also play a part in the evolution of the disease that is caused by HPV [8]. These other risk factors most likely affect the immune surveillance or act as extra carcinogens. 8 A tiny percentage of infections are responsible for the formation of low-and/or high-grade cervical intraepithelial neoplasia (CIN), which, despite having a long period of latency, can still regress or advance to invasive cervical cancer. The primary effects of HPV oncogenes include the following: the degradation of p53 by E6 and, as a result, the inhibition of apoptosis, which results in the prolonged survival of cells that have been heavily damaged and altered; the elevation of the protein ki 67, which serves as a proliferation marker; and the release of E2F from pRb by E7, which results in the ongoing activation of the cell cycle. This Rb-E2F pathway is controlled by a group of molecules called cyclin-dependent kinase inhibitors, one of which is called p16 [9-11]. These molecules prevent enzymes from phosphorylating pRB (cyclin-dependent kinases). The activation of p16 does not have any downstream consequence in cells that are infected with HPV that can convert. As a direct consequence of this, p16 is overexpressed to an extreme degree and builds up in the cells. Overexpression of p16 has been observed in the majority of precancers and malignancies of the cervix, in contrast to the extremely rare occurrence of p16 expression in normal tissue [12].

In the conduct of our research, we set out with the goal of locating and validating certain markers that may be used to identify cervical cells that exhibit the HR-HPV oncogenes E6-E7 and have maintained the ability to both replicate their cellular genomes and proliferate. It was hypothesised that these cells would exhibit a high level of genetic instability and thus provide a pool from which cervical neoplasms would originate [13]. We used Mouse monoclonal antibodies from the Biogenex Life systems Histology Kit to research the importance of p16 and Ki 67 in the diagnosis of cervical lesions. The research was carried out on a total of 80 cervical biopsy specimens. The study by Das *et al*. included 545 female participants with a mean age of 36.8 years. 9 patients out of a total of 545 who satisfied the inclusion criteria did not have cytology smears that were adequate for examination [14].

The incidence rate of HPV infections dropped significantly as people became older. At the time of enrolment, HPV was found in 29.7 percent of the women in the age range of 21-24 years, 9.7 percent of the women in the age group of 40-49 years, and 6.0 percent of the women older than 60 years.

According to a study by Stoler *et al*., the overall prevalence of HPV cases decreased with

increasing age. At the time of enrolment, HPV was found in 29.7 percent of the women in the age range of 21-24 years, 9.7 percent of the women in the age group of 40-49 years and 6.0 percent of the women older than 60 years [15]. According to the findings of our research, the age range of 41 to 60 years was associated with the highest frequency of premalignant and malignant cervical lesions. Kalyani *et al*. found a comparable age distribution in their research [16]. Liu *et al*., study ages of the patients ranged anywhere from 18 to 73 years old (mean 39). Initially, these cases were reported as benign (n=88), LSIL/CIN 1 (n=90), HSIL/CIN 2 (n=35) and HSIL/CIN 3 (n=7) by a total of five different pathologists [17].

According to the findings of Aswathy *et al*. [18], the age range of 35-50 years was the one that was most frequently affected by cancer of the cervix. Consequently, the age of the patient is the primary risk factor for acquiring cervical cancer. The fact that even the youngest participant in our study, who was just 21 years old, had CIN II demonstrates the importance of performing PAP screening tests at an early age.

Squamous cell carcinomas accounted for around 87 percent of all cervical malignancies, whereas adenocarcinomas made for the remaining 13 percent. Studies carried out by Das B.C. *et al*. [19] and Misra *et al*. [20] came to similar conclusions, indicating that approximately 85–90 percent of cases are squamous cell carcinoma, with the remaining cases being adenocarcinoma. These findings have been confirmed by subsequent research. This could be because of the higher incidence of cervical cancer and, as a result, increased awareness of detecting early CIN lesions, which has occurred in countries like India. On the other hand, patients in other countries may present with advanced lesions.

Location of staining

In our research, we found that a combination of nuclear and cytoplasmic staining was present in the majority of instances of LSIL, HSIL, and invasive malignancy. This was an unusual finding given that only a small percentage of LSIL cases displayed focal weak cytoplasmic staining. We did not come across even a single instance of particularly intense nuclear staining. Since P16 is a nuclear protein, its presence in the cytoplasm may be the result of a form of post-transcriptional modification or, more simply, an excessive amount of the protein may drive its transfer into the cytoplasm. Both of these possibilities are possible. According to the research carried out by Murphy *et al*. 15, p16INK4A expression was found to be mostly nuclear in CIN1 cases, cytoplasmic in CIN2, CIN3, cGIN, and invasive cases, and both nuclear and cytoplasmic in invasive instances [21].

Comparison of p16 immunostaining in normal cervix
chronic cervicitis (Negative control) with other studies

In total, 22 cases with a normal cervix were analyzed during the period of our research. Because normal cervical epithelium did not express p16 INK 4a, it was used as a negative control for the experiment.

They proposed that p16INK4A expression in the normal squamous epithelium was seen particularly in the basal cell layer, and its positivity rate was lower (12.5 percent) as compared with CIN (75 percent) or SCC. The results of our study are consistent with those of Klaes *et al*. (2001) [18], Murphy *et al*. (2003) [22] and Redman *et al*. (2008) [23]. They found that p16INK4A expression in normal (75 percent).

According to a study conducted by Shi *et al*., [24] P16 was either not expressed at all in common cervical tissues or was expressed in a very small amount in LSIL tissues. On the other hand, P16 protein was expressed in HSIL tissues in the 1/3 to 2/3 layers of the cervical squamous epithelium, as well as in SCC tissues. P16 expression was found in the cervix's whole squamous epithelium, indicating that the intensity of P16 expression was highly linked

with the degree of cervical tissue lesions.

In the research carried out by Kumari *et al*. [25], four patients tested positive for p16 INK4A but were negative for intraepithelial lesion or malignancy (NILM). Investigators reinvestigated the slides that had been stained with haematoxylin and eosin (H&E), and after doing so, they classed these four cases as CIN1, CIN2 and CIN3. In many instances, the histopathological results were very mild, and the p16 immunostaining was the only method that was able to pick them up. This highlights the necessity of utilizing the p16 marker while doing cervicitis tests.

Comparison of p16 immunostaining in squamous metaplastic cells with other studies

In our research, squamous metaplastic cells displayed p16 negative (0/17) similar to Aslani *et al*. [26] Lori Iaconis *et al*., (2007) [27] discovered that 54 percent of cases of atypical immature metaplasia (AIM) were negative for both p16 and Ki-67, indicating benign reactive atypia. Two AIM cases (5%) came back negative for p16 but positive for Ki-67 in the area beside an ulcer, showing regeneration. I

Comparison of p16 immunostaining in LSIL (CIN I) cases with other studies

In our study, 16 of 25 patients with Low grade squamous intraepithelial lesion tested positive. Our findings are congruent with those of Hu *et al*. [28].

The absence of p16 in LSIL lesions could be related to a transitory infection with a low-risk HPV virus whose DNA is not incorporated into the cells. Less p16 expression is associated with low-risk HPV. Such instances may regress and necessitate close monitoring. In our study, 16/25 cases of LSIL had a higher score of 6, most likely due to HR HPV infection and there is a strong likelihood that it may progress to higher grades-CIN II and III-indicating the need for additional therapy. According to the findings of Zhang *et al*. [22], 51 (32 percent) of the 157 cases originally categorised as CIN 1 were reclassified as metaplasia based on "consensus" pathology.

A possible explanation for some significantly lower percentages of p16INK4a immunopositivity in low-grade lesions is that a certain percentage is thought to be caused by low-risk HPV types (about 20% of low-grade lesions in the ASCUS/Low-grade Triage Study were negative for high-risk HPV types). Overexpression of p16INK4a would not occur because the affinity of the E7 protein of low-risk HPV for Rb is significantly lower than that of high-risk PHV types.

Comparison of p16 immunostaining in HSIL (CIN II and CIN III)cases with other studies

In our study, all 13 cases diagnosed as High grade squamous intraepithelial lesions showed moderate to strong, both nuclear and cytoplasmic p16 positivity. The results of our study are concordant with that of Klaes *et al*. showed 70 to 98% rates of immunopositivity [22].

Comparison of present study with other studies of rates of P16INK4A Immunopositivity in SCC cases

Concerning cases diagnosed as Squamous cell carcinoma (n= 17), all (17/17) showed strong p16 positivity. Our results are similar to studies done by Srivatsava [30], Kumari *et al*. [25] and Wei *et al*. [31] showed 85% to 98% rates of immunopositivity.

Comparison of present study with other studies of rates of P16INK4A Immunopositivity in adenocarcinoma cases

In our study, 100 % of Adenocarcinoma (n= 3) cases were positive for p16.
Our study results are concordant with that of Murphy *et al*. [21] (2005) which showed 10/10, This suggests that HPV also plays a key role in the carcinogenesis of adenocarcinoma.

Comparison of grading system of p16

When comparing our results with that of Lesnikova *et al*. [32] who used the same semi-quantitative immune-histochemical scoring system which we have adapted, our immune-histochemical distribution of p16INK4A in normal and neoplastic tissue of the cervix were mostly similar.

Comparison of p16 IHC grading of reference study with present study

The immuno-histochemical score was used to evaluate the expression of p16INK4A in tissue microarray cores from 806 cervix biopsies encompassing normal tissues, CIN1, CIN2, CIN3, and invasive cervical cancer (ICC). Normal cervical epithelium in both investigations had consistently low immunohistological scores ranging from 0 to 2. p16INK4A immunohistological scores were high in specimens with dysplastic cervical epithelium, and these increased with CIN grade. However, a small but significant percentage of specimens with lower grades of dysplasia exhibited p16INK4Ascores (0-2) that were low or negative (9 percent of CIN1 and 5 percent of CIN2 lesions in Lesnikova *et al*. [32] study, 3 percent of CIN I in our study). In our investigation, 10/10 CIN II cases tested positive. Thus, it indicates that p16 expression increases as we progress from normal cervical epithelial to dysplasia of varying severity to cancer (both in intensity and proportion). As a result, it aids in the grading of cervical tumours for appropriate treatment.

KI 67 Immunostaining

The ki-67 antibody reacts with the nuclear Ki-67 antigen found in proliferating epithelium. Nicolas Wentzensen *et al*. revealed that p16/Ki-67 positive increased with histologic severity, from 26.8% in normal histology to 92.8 in CIN,93% of normal patients were Ki-67 negative, demonstrating only basal positive. The ki 67 positives in the normal cervix may be attributed to underlying reactive or regenerative activity [33].

That of histologic type

All 22 cases of cervicitis in our investigation tested negative for p16 and ki 67, demonstrating the adverse correlation of these markers with the normal cervix. Ki 67 positive was found in 20% of two cases of cervicitis with hyperplastic ectocervix. Out of 25 CIN I patients, 16 had p16 positivity and 19 had ki 67 positivity.
As a result, the use of Ki-67, p16, or even HPV testing to manage suspected LSIL should be confined to cases where the diagnosis is deemed required. According to the outcomes of the Magkana *et al*., study, when HPV16/18 and p16/ki-67 were negative, 3.6 percent of women with CIN2+ were missed, whereas when HPV 16/18 (+)/p16/ki-67 (+), all CIN2+ were detected [34]. In our investigation, the specificity of p16 was 100% and that of ki 6 was 90%. The sensitivity of p16 and ki67 in detecting LSIL is 65% and 75%, respectively, although both demonstrated 100% sensitivity in detecting HSIL and carcinomas. Supriya (2010) [23] stated in her study that because the p16 marker has an 80% sensitivity for LSIL, it should be

tested alongside the MIB-1 or HPV test. According to Sagasta *et al*., p16 was diffusely positive in 230 LSIL/CIN1 lesions (45%), focally positive in 123 (24%) and negative in 154 samples (30%); additionally, it has very little or no significance as a predictor of progression of LSIL/CIN1 in clinical practice [35].

Our findings also confirmed that p16 immunohistochemical staining is diffusely and highly positive in a small percentage of LSIL cases (16/25). The results revealed that p16 expression differed considerably across normal, LSIL, and HSIL specimens. Ki67 expression was also 5X different across normal, LSIL, and HSIL specimens. According to the Magkana study, p16/ki-67 is a safe and quick assay that might be used to identify CIN2+ among women with modest cervical lesions, with good sensitivity and specificity, thereby reducing the psychological and economic burden of HPV screening. It has been claimed that the biomarkers p16 and ki 67 cannot discriminate between LSIL and HSIL. Histologically, this differentiation must be made. The temptation to move lesions from LSIL to HSIL due to strong p16 immunostaining should be avoided [34]. In many circumstances, the therapeutic significance of this diagnosis is uncertain. In the clinical setting, biomarkers should be used only when diagnostic precision is critical 13.

Conclusion

Squamous cell carcinoma is the most frequent type of carcinoma cervix in women aged 40-60. P16 INK4a-negative low-grade cervical lesions are associated with low-risk HPV and may resolve over time. Diffuse p16INK4a staining in CIN I indicates a higher risk for recurrence of high-grade precancer or invasive lesions, which necessitates continued therapy. In the cancer cervix, p16 and ki 67 were upregulated, and their expression increased with CINs/SILs severity. Correlation between p16 and ki 67 grades in cervical neoplasia demonstrated increased p16 expression with consistently increasing ki 67 in increasing severity groups and is more accurate and consistent in higher grades. P16 detects early dysplasia more precisely than ki 67 (regenerative/reactive atypia also tests positive). Both markers detect high-grade dysplasia and carcinomas more sensitively than LSIL lesions. LSIL and HSIL biomarkers don't differentiate. In practice, biomarkers should be implemented when diagnostic precision is clinically significant. The present findings can be used as a guideline for future research on p16 and ki-67 in cervical cancer. P16 and ki 67 are biomarkers for CIEN. Diffuse p16 expression can be a surrogate sign of changed high-risk HPV-related cervical lesions, assisting pathologists to distinguish lesions which need follow-up testing.

Disclosure: None declared.

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